Forkhead transcription factors, Fkh1p and Fkh2p, collaborate with Mcm1p to control transcription required for M-phase

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Background: The 'CLB2 cluster' in *Saccharomyces cerevisiae* consists of approximately 33 genes whose transcription peaks in late G2/early M phase of the cell cycle. Many of these genes are required for execution of the mitotic program and then for cytokinesis. The transcription factor SFF (SWI5 factor) is thought to regulate a program of mitotic transcription in conjunction with the general transcription factor Mcm1p. The identity of SFF has yet to be determined; hence further understanding of the mechanisms that regulate entry to M phase at the transcriptional level requires characterization of SFF at the molecular level.

Results: We have purified the biochemical activity corresponding to SFF and identified it as the forkhead transcription factor Fkh2p. Fkh2p assembles into ternary complexes with Mcm1p on both the *SWI5* and *CLB2* cell-cycle-regulated upstream activating sequence (UAS) elements *in vitro*, and in an Mcm1p-dependent manner *in vivo*. Another closely related forkhead protein, Fkh1p, is also recruited to the *CLB2* promoter *in vivo*. We show that both *FKH1* and *FKH2* play essential roles in the activation of the CLB2 cluster genes during G2–M and in establishing their transcriptional periodicity. Hence, Fkh1p and Fkhp2 show the properties expected of SFF, both *in vitro* and *in vivo*.

Conclusions: Forkhead transcription factors have redundant roles in the control of CLB2 cluster genes during the G2–M period of the cell cycle, in collaboration with Mcm1p.

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Background

The eukaryotic cell cycle is controlled so that the temporal order of DNA replication, nuclear division and cytokinesis takes place in a well-defined and reproducible order. The periodic activation of cell-cycle regulatory genes at the transcriptional level is fundamental to these controls and has been particularly well characterized for the G1 to S-phase transition in the budding yeast *Saccharomyces cerevisiae* [1]. Microarray analysis has identified several waves, or clusters, of transcriptional activity associated with cell-cycle progression [2]. The best characterized of these, the 'G1 cluster', is composed of genes required for entry and passage through S phase. Two transcriptional pathways regulate this cluster. The first depends on the Swi4p and Swi6p transcription factors which bind as a heterodimer to SCB elements in promoter regions of the *CLN1,2* and *HO* genes [1]. The second pathway uses Swi6p, but with a different heteromeric binding partner, Mbp1p. Swi6p–Mbp1p heterodimers bind and activate through MCB elements in the promoter regions of genes required for S-phase progression and, together with SCB-regulated genes, trigger events following START, such as bud emergence, spindle pole duplication and DNA replication. Over 75 genes in budding yeast, collectively known as the G1 cluster, show a clear G1 expression profile characteristic of those under MCB/SCB control [2].

Many of the key regulatory genes required for the G2–M transition and then for mitotic progression and cytokinesis are also cell-cycle-regulated and together make up the 'CLB2 cluster'. Of the 30 or so CLB2 cluster genes, the best studied are *SWI5* and *CLB2*, for which it has been shown that the periodicity of their respective transcripts is dependent on the general regulator of transcription Mcm1p [3–5]. Mcm1p is an essential sequence-specific homodimeric DNA-binding protein that is a member of the MADS box transcription factor family [6]. It has well-defined roles in the control of genes that determine cell-type identity [6], general metabolism [7], minichromosome maintenance [8], and in M-G1 [9] and G2–M transcription [3–5]. Mcm1p achieves its functional versatility through the recruitment of specific co-activators and co-repressors to different promoters by protein–protein interactions.

As Mcm1p has no reported cell-cycle-regulated activity, it is likely that any cell-cycle-regulated transcription

involving Mcm1p will involve other specialized factors. This possibility was first suggested from studies of the *SWI5* promoter, where an upstream activating sequence ($SWI5_{UAS}$), both necessary and sufficient for G2–M-regulated transcription, was shown to be dependent on Mcm1p and a previously uncharacterized factor, SFF [3]. A similar Mcm1p-dependent regulatory element has also been characterized in the *CLB2* promoter [5]. In the case of $SWI5_{UAS}$, a single Mcm1p site is present, whereas for the $CLB2_{UAS}$, three Mcm1p sites spaced over approximately 250 base pairs are required for optimal promoter activity [5,10], although a single site can function with reduced activity *in vivo*. In addition to Mcm1p, SFF is recruited to $SWI5_{UAS}$ and $CLB2_{UAS}$ *in vitro*. Although SFF makes base-specific contacts with $SWI5_{UAS}$ *in vitro*, indicative of direct DNA binding [3,4], it is reliant on Mcm1p for this function and is incapable of autonomous DNA binding to this site. A role for SFF in the control of *SWI5* and *CLB2* transcription is suggested from the observation that a single base mutation in $SWI5_{UAS,}$ where SFF makes specific base contacts, results in loss of SFF binding *in vitro* and abolishes UAS activity *in vivo* [3]. Although Mcm1p is a polypeptide of 286 amino acids, its essential functions require only the 96 amino-terminal residues [11–13]. This domain contains all the necessary sequences for homodimerization, DNA binding and the recruitment of different accessory proteins, including SFF, to promoters.

Although an extensive molecular analysis of CLB2 cluster genes has not been performed, results for those studied indicate that Mcm1p and SFF are required for the periodic expression of these genes during the G2–M transition. Moreover, potential binding sites for Mcm1p and SFF have been identified in the vast majority of CLB2 cluster promoters [2], further supporting the possibility that Mcm1p and SFF coordinate this wave of transcription. The general activation of genes during G2–M progression requires the activity of the cyclin-dependent kinases Cdk1p (Cdc28p)/Clbp, suggesting that Clbp-associated kinase activity is involved in a positive feedback loop whereby Clb activity is required for *CLB* transcription [14]. This could occur through direct regulation of the Mcm1p–SFF complex and, as such, offers a potential mechanism by which transcription of the CLB2 cluster could be regulated.

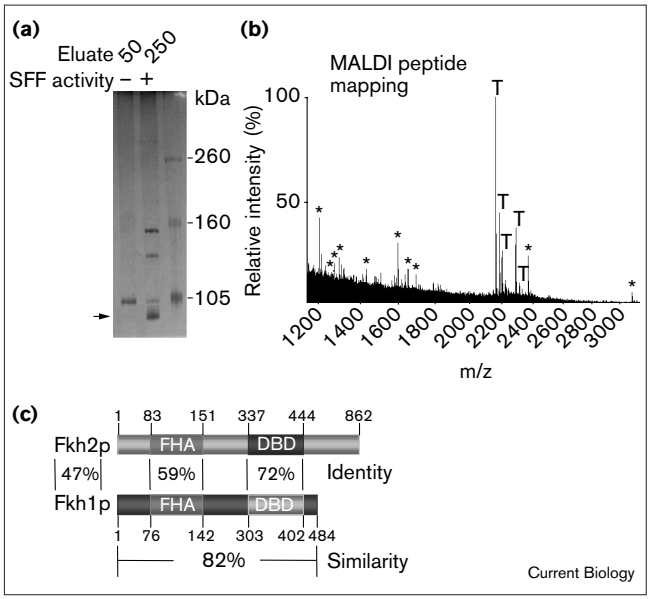
We report here the purification, identification and molecular characterization of the activity previously described as SFF [3–5]. We show that Fkh2p, a winged-helix transcription factor, corresponds to the activity previously shown to form Mcm1p-dependent ternary complexes on the cell-cycle-regulated $SWI5_{UAS}$ and $CLB2_{UAS}$ elements. Our data shows that Fkh2p and the related forkhead family member, Fkh1p, have overlapping and redundant roles in the control of CLB2 cluster transcripts in budding yeast.

Results

Biochemical purification of SFF

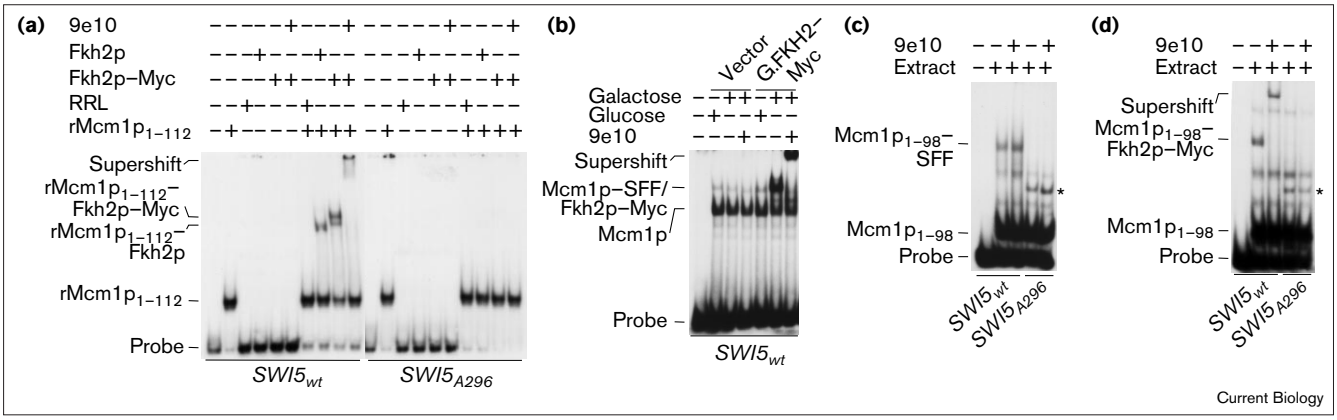
The activity corresponding to SFF was purified from yeast cell extracts by two rounds of affinity chromatography, using affinity beads generated by concatenating $SWI5_{UAS}$ oligonucleotide duplexes (6mers) that were biotinylated and then coupled to streptavidin beads. The identification of SFF and Mcm1p activities in column fractions were followed by band-shift analysis as described previously [5], and purified SFF activity was resolved by one-dimensional PAGE and silver staining (Figure 1a). Several polypeptides co-eluted with the major fraction of SFF activity (250 mM NaCl eluate), but our attention focused on a polypeptide of 98 kDa. This was of particular interest because we had previously identified a polypeptide of similar molecular mass that could be UV crosslinked to a $SWI5_{UAS}$ oligonucleotide duplex in band-shift assays (our unpublished data). This band (Figure 1a) was excised

Figure 1



Identification of Fkh2p as an SFF-like DNA-binding activity. **(a)** Affinity purification of SFF activity from whole-cell extracts. SFF activity was tracked by band-shift analysis throughout two rounds of affinity purification using $SWI5_{UAS}$ affinity beads. 50 mM and 250 mM eluates from second-round purifications were resolved by SDS–PAGE and polypeptides were visualized by silver staining. The candidate SFF polypeptide (arrow) migrated at approximately 98 kDa (inferred from previous UV crosslinking analysis). **(b)** Protein identification by high-mass accuracy MALDI peptide mapping. A 0.5 µl aliquot was withdrawn from a supernatant of the in-gel digest and analyzed by MALDI mass spectrometry. Masses of 10 peptide ions matched the masses of corresponding tryptic peptides from the Fkh2p protein (designated by asterisks) with accuracy better than 150 ppm and covered 17% of the total protein sequence. T, autolysis products of trypsin. **(c)** The modular structure and similarity of the Fkh1p and Fkh2p forkhead transcription factors are shown, highlighting the forkhead-associated domain (FHA) and the forkhead DNA-binding domain (DBD). The percent amino-acid identity and similarity is indicated.

Figure 2



Ternary complex formation by Fkh2p *in vitro*. **(a)** The ability of *in vitro* translated Fkh2p, or epitope-tagged Fkh2p (Fkh2p–Myc), to form rMcm1p$_{1-112}$-dependent ternary complexes on the *SWI5*$_{UAS}$ was tested. Wild-type (*SWI5*$_{wt}$) or mutant (*SWI5*$_{A296}$) probe was used in band-shift assays with, or without, rMcm1p$_{1-112}$ and either Fkh2p, Fkh2p–Myc or unprogrammed rabbit reticulocyte lysate (RRL). Free probe and the position of complexes containing rMcm1p$_{1-112}$, rMcm1p$_{1-112}$–Fkh2p/Fkh2p–Myc and supershifted complexes are indicated. The addition of anti-Myc monoclonal antibody 9e10 to assays is indicated and the resulting supershift activity is shown. Fkh2p translated in RRL is full-length polypeptide: amino acids 1–862 (Fkh2p) or 1–862–Myc (Fkh2p–Myc). **(b)** Wild-type cells (*W303*: *MAT**a** MCM1 ura3–52*) were transformed with pYES2 vector alone or the corresponding galactose-inducible *FKH2–MYC* expression plasmid, pG.FKH2–MYC. Asynchronous log-phase cells were grown in raffinose-containing medium that was adjusted to either 2% glucose or 2% galactose. After a further 2 h, extracts were prepared and used for band-shift analysis with *SWI5*$_{wt}$ probe. Identification of Fkh2p–Myc as the induced band present in Mcm1p–SFF complexes was shown by addition of 9e10 to the band-shift reaction. The resulting supershift activity is indicated. **(c)** Cell extracts prepared from a *W303* background expressing truncated Mcm1p$_{1-98}$ (*W303*: *MAT**a** mcm1::LEU2 ura3::ADH1-mcm1*$_{1-98}$) were included in band-shift assays with *SWI5*$_{wt}$ or *SWI5*$_{A296}$ probe. The formation of Mcm1p$_{1-98}$, and Mcm1p$_{1-98}$–SFF complexes with probe are indicated. Addition of 9e10 does not shift any of the complexes detected (compare with (d)), and on the mutant probe an uncharacterized complex appears (indicated by an asterisk). **(d)** Extracts were prepared from a strain expressing epitope-tagged Fkh2p–Myc (*W303*: *MAT**a** mcm1::LEU2 ura3::ADH1-mcm1*$_{1-98}$ *fkh2::FKH2–MYC-TRP1*) and included *SWI5*$_{wt}$ or *SWI5*$_{A296}$ probe as described in (c). The formation of Mcm1p$_{1-98}$, and Mcm1p$_{1-98}$–Fkh2p–Myc complexes with probe are indicated. Addition of 9e10 and the position of mutant-specific complex (indicated by an asterisk), and of the supershifted complex, are indicated.

from the silver-stained gel and characterized further by mass spectrometry MALDI analysis (Figure 1b). This led to the identification of the polypeptide as the forkhead transcription factor Fkh2p. Although several forkhead transcription factors have been identified in *Saccharomyces cerevisiae* [15,16], only one of these, Fkh1p, has significant sequence similarity to Fkh2p outside of the forkhead DNA-binding domain (Figure 1c). This similarity includes the forkhead-associated (FHA) domain [15], which is found in a subset of the forkhead family.

Fkh2p is an SFF-like ternary complex factor

To confirm that Fkh2p had SFF-like DNA-binding activity, we first tested its ability to form ternary complexes on *SWI5*$_{UAS}$ in an Mcm1p-dependent manner. Fkh2p was translated in rabbit reticulocyte lysate and its ability to bind *SWI5*$_{UAS}$, either in the presence or absence of recombinant Mcm1p (rMcm1p$_{1-112}$), was tested by band-shift analysis (Figure 2). In the presence of rMcm1p$_{1-112}$, Fkh2p was able to form ternary complexes on the *SWI5*$_{UAS}$ probe but was unable to bind readily in the absence of rMcm1p. Only after long exposure times could any sign of autonomous Fkh2p binding (Mcm1p-independent) to *SWI5*$_{UAS}$ be detected (data not shown). The recruitment of Fkh2p into ternary complexes with rMcm1p, like that of SFF, was also abolished by introducing a single base substitution, an adenine at position 296, into the *SWI5*$_{UAS}$ probe to give *SWI5*$_{A296}$. The reconstituted ternary complexes which form on the *SWI5*$_{UAS}$ *in vitro* (Figure 2a) therefore behave in a manner indistinguishable from the SFF activity previously characterized in cell extracts ([3], and Figure 2c).

To further characterize Mcm1p–Fkh2p complexes, sequences encoding six concatenated Myc epitopes, which can be detected by the monoclonal antibody 9e10, were fused to the carboxyl terminus of the FKH2 open reading frame (ORF). This fusion, *FKH2–MYC*, was placed under the control of the galactose-inducible promoter *GAL1* in a high-copy number expression construct, pG.FKH2–MYC. Cells transformed with vector alone or those grown on glucose with pG.FKH2–MYC display typical Mcm1p and Mcm1p–SFF ternary complexes in band-shift analysis (Figure 2b). Induction of *FKH2–MYC* after addition of galactose stimulated the formation of additional ternary complex on the *SWI5* probe which was almost completely supershifted by addition of antibody

9e10 (Figure 2b). This shows that Fkh2p can assemble into SFF-like complexes with Mcm1p in cell lysates.

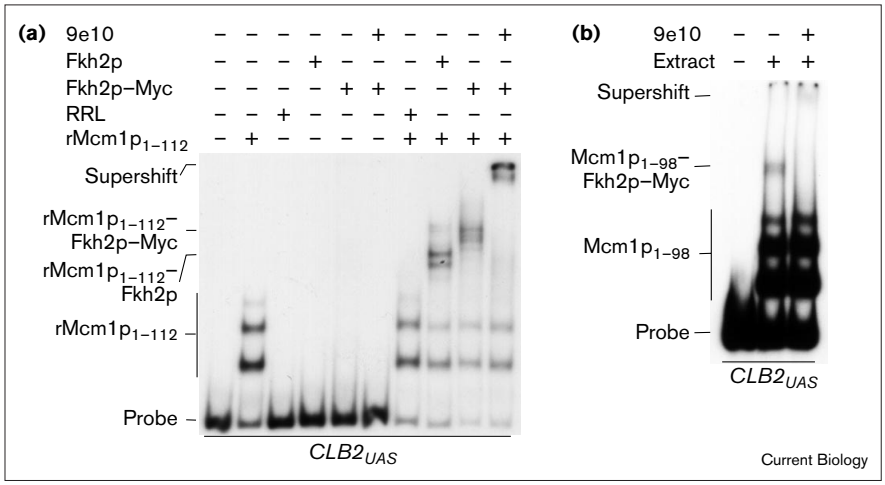
Figure 2c,d shows ternary complex formation on a $SWI5_{UAS}$ probe generated from extracts expressing $Mcm1p_{1–98}$ and either untagged Fkh2p (Figure 2c) or Fkh2p–Myc (Figure 2d), respectively. For the experiment shown in Figure 2d, the epitope-tagged Fkh2p is derived from a strain where the *FKH2–MYC* fusion replaces *FKH2* at its own locus and thus *FKH2–MYC* expression is under control of the authentic *FKH2* promoter. Band-shift complexes formed using extracts from *FKH2* and *FKH2–MYC* strains display similar patterns, including complexes containing Mcm1p only and those where SFF activities are recruited into ternary complexes. To demonstrate that Fkh2p is a component of SFF activity in these cell extracts, antibody 9e10 was added to assays, resulting in a complete supershift of ternary complexes in the *FKH2–MYC* strain (Figure 2d) but not in the isogenic *FKH2* strain. Hence, the supershift is specific for Fkh2p–Myc, thus demonstrating that Fkh2p is a major component of SFF. The SFF activities in *FKH2* and *FKH2–MYC* strains were both sensitive to the $SWI5_{A296}$ mutation (Figure 2c,d), indicating that they had consistent DNA-binding activities. We also note the appearance of an additional band (marked with an asterisk in Figure 2c,d) specific to the mutant probe. The identity and significance of this species has not yet been determined.

We and others [4,5,10], have previously characterized a cell-cycle-regulated UAS in the *CLB2* promoter ($CLB2_{UAS}$) that requires Mcm1p and an SFF-like ternary complex factor for activation of *CLB2* transcription during G2–M phase. To determine if Fkh2p could also assemble into complexes with Mcm1p at the $CLB2_{UAS}$ *in vitro*, similar assays were used to those described for the characterization of $SWI5_{UAS}$ (see Figure 2). Fkh2p translated *in vitro* was shown to form complexes at $CLB2_{UAS}$ in an Mcm1p-dependent fashion (Figure 3a) and, as seen for the *SWI5* UAS, displayed only a low level of autonomous DNA-binding activity (data not shown). Multiple Mcm1p-dependent ternary complexes were detected when Fkh2p was added in these assays. This probably reflects the ability of the $CLB2_{UAS}$ to recruit multiple Fkh2p molecules, consistent with the identification of three Mcm1p consensus binding sites in the probe. A similar band-shift pattern was seen on the $CLB2_{UAS}$ probe when cell extracts were used in place of recombinant protein. Using the *FKH2–MYC* strain, we show that the slow-migrating complex formed on the *CLB2* probe is supershifted by addition of antibody 9e10, indicating that Fkh2p–Myc is assembled into higher-order complexes on the $CLB2_{UAS}$ probe (Figure 3b). This complex is not recognized by 9e10 in the equivalent complexes generated from the untagged *FKH2* strain (data not shown). Although we have not formally demonstrated this point, it is possible that, as with the reconstituted complexes (Figure 3a), multiple Mcm1p homodimers are loading onto the $CLB2_{UAS}$.

Mcm1p-dependent recruitment of Fkh2p to the *SWI5* and *CLB2* promoters *in vivo*

To determine whether Fkh2p is recruited into complexes at the *SWI5* and *CLB2* promoters *in vivo*, we performed

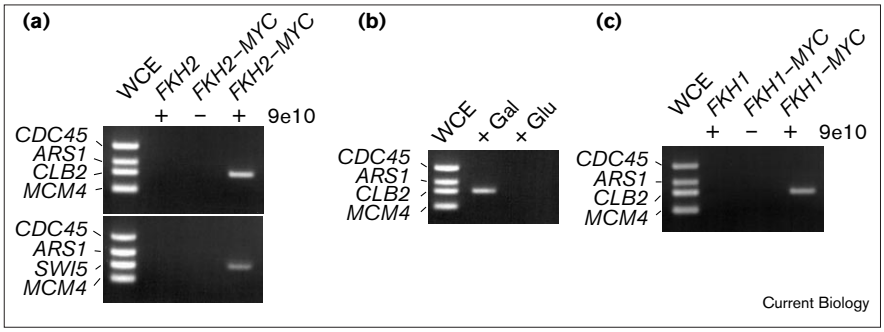
Figure 3



Assembly of Fkh2p into complexes with the $CLB2_{UAS}$ *in vitro* is dependent on Mcm1p. **(a)** $CLB2_{UAS}$ probe spanning the −362 to −131 region of the *CLB2* promoter [5] was used in band-shift assays with or without $rMcm1p_{1–112}$ and either Fkh2p, Fkh2p–Myc or unprogrammed RRL. Free probe and the position of complexes containing $rMcm1p_{1–112}$, $rMcm1p_{1–112}$–Fkh2p/Fkh2p–Myc and supershifted complexes are indicated. The addition of anti-Myc-antibody 9e10 is indicated and the resulting supershift activity is shown. Multiple $rMcm1p_{1–112}$-dependent complexes are present, presumably because of the presence of multiple Mcm1p-binding sites in the probe. **(b)** Cell extracts were prepared as described in Figure 2d from a strain expressing epitope-tagged Fkh2p (*W303*: *MAT**a*** *mcm1::LEU2 ura3::ADH1-mcm1$_{1–98}$ fkh2::FKH2–MYC-TRP1*). Band-shift analysis was performed with a $CLB2_{UAS}$ probe as described in (a). 9e10 was added to assays where indicated, generating a supershifted complex.

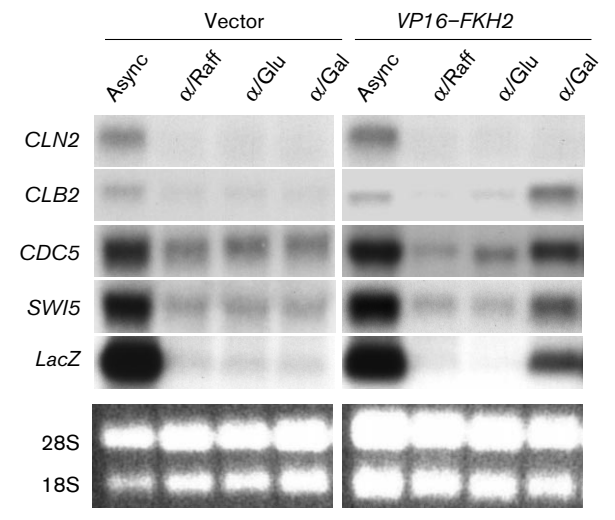
chromatin immunoprecipitation (CHIP) assays on formaldehyde cross-linked chromatin, using the *FKH2–MYC* strain. Following immunoprecipitation of chromatin with 9e10, the presence of various DNA fragments was determined using PCR and gene-specific primer pairs. In the case of *SWI5* and *CLB2*, primers were designed to enable detection of chromatin corresponding to the cell-cycle-regulated $SWI5_{UAS}$ and $CLB2_{UAS}$ elements. Additional primer pairs were included that amplify 5′ flanking regions of genes outside of the CLB2 cluster (*CDC45*, *MCM4*) and the *ARS1* replication origin. Following PCR amplification, we detected specific association of Fkh2p–Myc with *SWI5* and *CLB2* promoters but not with *CDC45*, *MCM4* or *ARS1* (Figure 4a). To test if the association of Fkh2p–Myc *in vivo* was dependent on Mcm1p, chromatin was prepared from a *Δmcm1 FKH2–MYC* strain where Mcm1p was generated from a plasmid that expressed an unstable ubiquitinated derivative (Ubi-$Mcm1p_{1–98}$ [4]). The Ubi-$Mcm1p_{1–98}$ was expressed from the galactose-inducible promoter (*GAL1*) so that cell viability could only be maintained on galactose. It has previously been shown that a shift to glucose results in the rapid disappearance of Ubi-Mcm1p, hence depleting the cell of functional Mcm1p. We show that when cells are grown on galactose under these experimental conditions, Fkh2p can be seen to associate with the *CLB2* promoter. Upon shift to glucose for 3 hours, this association was lost (Figure 4b). These data are consistent with previous findings that depletion of Mcm1p results in the shutdown of *CLB2*, *SWI5* and *CDC5* transcription and that Mcm1p is required for the assembly of ternary complexes on the *CLB2* promoter *in vivo* [4]. The ability of Fkh1p to associate with the *CLB2* promoter was also tested using a *FKH1–MYC* strain under the conditions described for Fkh2p. We showed that Fkh1p–Myc can specifically associate with the *CLB2* promoter *in vivo* (Figure 4c). The Mcm1p-dependency of this association was not tested. Our data shows that Fkh2p and Fkh1p are recruited to promoters of the CLB2 cluster *in vivo*. As a final demonstration of this, we tested the ability of a Vp16–Fkh2p fusion protein, in which Fkh2p is fused to the acidic transcriptional activation domain of the herpes simplex virus Vp16 protein, to activate these genes at times in the cell cycle when transcription of the CLB2 cluster is low, such as in G1. The logic behind this experiment was that if Fkh2p can bind CLB2 cluster promoters throughout the cell cycle (as appears to be the case for SFF [4]), when fused to a constitutive *trans*-activation domain, it should activate transcription throughout the cell cycle instead of being confined to late G2–M phase. To resolve this issue, we expressed a Vp16–Fkh2p fusion protein from a galactose-inducible promoter and determined if this could activate the $CLB2_{UAS}$–*ubiYLacZ* reporter gene and a panel of endogenous genes including *CLN2*, *CLB2*, *CDC5* and *SWI5* in an α-factor-induced G1 arrest. In cells grown on raffinose (*GAL* promoter off) carrying vector alone or with the galactose-inducible *VP16–FKH2* expression vector, *CLN2*, *CLB2*, *CDC5*, *SWI5* and *lacZ* mRNAs were readily detected by northern blot analysis in asynchronous samples (Figure 5). In α-factor-arrested cells grown on raffinose and glucose, these transcripts remained repressed. When cells were shifted to galactose, however, transcripts for *CLB2*, *CDC5*, *SWI5* and *lacZ* were significantly increased in cells carrying the *VP16–FKH2* expression plasmid, in comparison to the situation on raffinose and glucose (Figure 5). This activation was judged to be specific for *CLB2*, *CDC5*, *SWI5* and *lacZ* because *CLN2* did not respond to *VP16–FKH2* induction. Together, these data support the conclusions from the *in vivo* chromatin immunoprecipitation assays (Figure 4) showing that Fkh2p is recruited to the *CLB2* promoter *in vivo*.

Figure 4



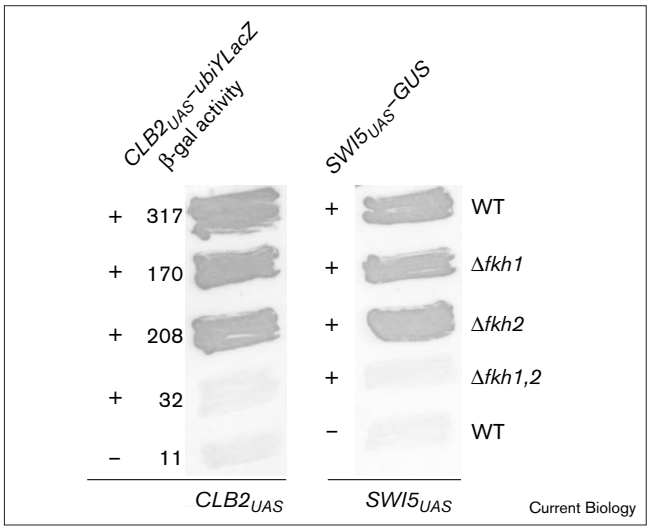
Fkh1p and Fkh2p are recruited to promoters of the CLB2 cluster genes *in vivo*. **(a)** *FKH2* or the *FKH2–MYC* strain was grown to mid-log phase and, following chromatin immunoprecipitation, DNA was amplified by PCR. Primers that specifically amplify regions of the *SWI5* (370 bp) or *CLB2* (380 bp) promoters overlapping with $SWI5_{UAS}$ and $CLB2_{UAS}$ elements were used in conjunction with primers that amplify flanking regions of the *CDC45* (490 bp), *MCM4* (300 bp) genes and the *ARS1* replication origin (440 bp). Where indicated, anti-Myc antibody 9e10 was either added or omitted. WCE, whole-cell extract. **(b)** Recruitment of Fkh2p to the *CLB2* promoter is dependent on Mcm1p. Chromatin crosslinked *in vivo* was immunoprecipitated from a *Δmcm1* strain carrying a *CEN*-based plasmid that expresses a ubiquitin–$Mcm1p_{1–98}$ fusion protein [4] under control of the *GAL1* promoter (W303: *mcm1::LEU2 fkh2::FKH2–MYC-TRP1 pG.ubi-mcm1*$_{1–98}$). PCR analysis using primer sets was as described in (a). Cells were continually grown in YEP galactose medium (+ Gal) or, 3 h before formaldehyde crosslinking, were washed and resuspended in YEP glucose (+ Glu). Cells were harvested during mid-log phase growth. **(c)** The experiment described in (a) was repeated with crosslinked chromatin from a *FKH1–MYC* strain.

Figure 5



A Vp16–Fkh2p fusion protein transcriptionally activates CLB2 cluster genes *in vivo*. Asynchronous cells carrying a $CLB2_{UAS}$-*LacZ* reporter gene (*W303*: *MAT**a** bar1 ura3::*$CLB2_{UAS}$-*ubiYLacZ* [5]) were grown in YEP + 2% raffinose to mid-log phase and then arrested in α-factor for 2 h. The UAS in this reporter contains three Mcm1p binding sites (–362 to –131) as described previously [5]. Cell cultures were split and adjusted to either 2% glucose (GAL promoter off) or 2% galactose (GAL promoter on) and then cultured for a further 2 h. mRNA samples were prepared and individual transcript levels analyzed by northern blot hybridization. Samples were prepared from cells carrying a low copy-number plasmid that expressed a fusion protein consisting of the acidic transcriptional activation domain of the herpes simplex virus Vp16 protein (amino acids 412–490), immediately followed by $Fkh2p_{1-457}$ under control of the *GAL1* promoter (pG.VP16-FKH2) or the vector alone. Blots were incubated with ^{32}P-labeled probes to detect *CLN2*, *CLB2*, *CDC5*, *SWI5* and *lacZ* mRNAs. 28S and 18S rRNA bands from these gels are shown to evaluate loading consistency.

Figure 6



Fkh1p and Fkh2p have redundant roles in the regulation of $CLB2_{UAS}$ and $SWI5_{UAS}$ activity *in vivo*. The activity of $SWI5_{UAS}$–*GUS* and $CLB2_{UAS}$–*ubiYLacZ* reporter genes were evaluated in *FKH1 FKH2*, *Δfkh1 FKH2*, *FKH1 Δfkh2* and *Δfkh1 Δfkh2* backgrounds (*W303*: *MAT**a** bar1 ura3::*$CLB2_{UAS}$–*ubiYLacZ p* $SWI5_{UAS}$–*GUS*). Qualitative plate assays were performed for $CLB2_{UAS}$–*ubiYLacZ* and $SWI5_{UAS}$–*GUS* reporter genes. Quantitative data for β-gal activity associated with the $CLB2_{UAS}$–*ubiYLacZ* reporter gene is also shown (average values of an experiment performed in triplicate).

Two forkhead genes, *FKH1* and *FKH2*, are required for *SWI5* and *CLB2* UAS activity

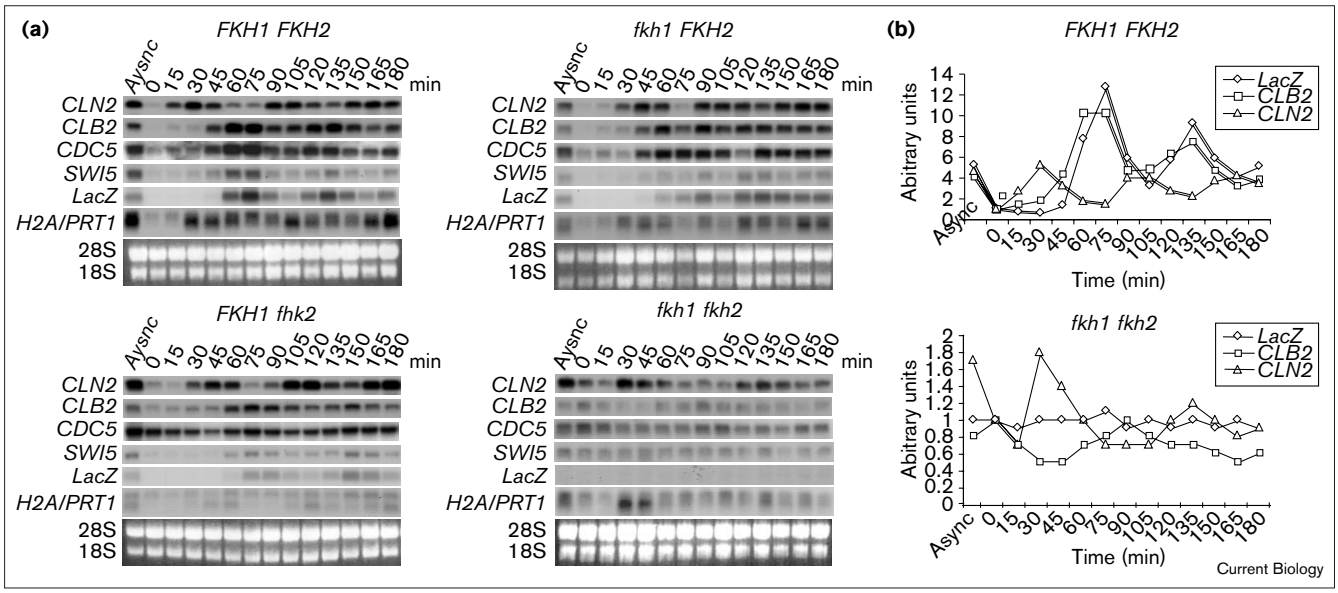
Activation of *SWI5* and *CLB2* transcription during G2–M is known to depend on specific UAS elements in the promoter regions of these genes [3–5,10]. For cell-cycle regulated activity, both UAS elements require Mcm1p and a ternary complex factor that we identify in this report as Fkh2p. To evaluate the possible role of *FKH2* in the transcriptional control of these genes, we compared the activity of $SWI5_{UAS}$ and $CLB2_{UAS}$ by their ability to drive expression of the *lacZ* and *GUS* (glucuronidase) reporter genes, respectively, in *FKH2* and *Δfkh2* genetic backgrounds. Using qualitative plate-based β-galactosidase and β-glucuronidase assays, no appreciable change in $CLB2_{UAS}$–*ubiYLacZ* or $SWI5_{UAS}$–*GUS* reporter activity was seen in the *Δfkh2* mutant, and in quantitative liquid assays only a modest decrease in $CLB2_{UAS}$–reporter activity was observed (Figure 6).

These results raised the possibility that Fkh2p may have a functionally redundant role in the control of *SWI5* and *CLB2* transcription. This implied the existence of a second factor that can functionally substitute for Fkh2p. The most likely candidate was the closely related forkhead protein, Fkh1p (see Figure 1c). To determine if Fkh1p and Fkh2p have overlapping roles in G2–M transcription, we constructed the single-deletion strain *Δfkh1* and the double-deletion strain *Δfkh1 Δfkh2*, and then determined UAS activity in the different genetic backgrounds. As predicted, deletion of both *FKH1* and *FKH2* was required to extinguish the activity of $SWI5_{UAS}$–*GUS* and $CLB2_{UAS}$–*ubiYLacZ* reporters, whereas single-deletion mutants (*Δfkh1* or *Δfkh2*) had negligible effect on UAS activity in both assays (Figure 6). The related transcription factors Fkh1p and Fkh2p therefore appear to have overlapping functions in the control of $CLB2_{UAS}$ and $SWI5_{UAS}$ activity.

Fkh1p and Fkh2p are required for cell-cycle regulation of transcription during G2–M

To define more rigorously the role of *FKH1* and *FKH2* in transcription of the CLB2 cluster genes, we analyzed transcript levels in α-factor-synchronized *FKH1 FKH2*, *Δfkh1 FKH2*, *FKH1 Δfkh2* and *Δfkh1 Δfkh2* backgrounds. Using northern blot hybridization, we evaluated transcript profiles across consecutive cell cycles for *CLB2*, *CDC5*, *SWI5*, *CLN2*, *H2A* and *PRT1* (Figure 7). To assess

Figure 7



FKH1 and *FKH2* are required for cell-cycle regulation of transcription during G2–M. **(a)** Cells with various forkhead genetic backgrounds and carrying a $CLB2_{UAS}$–*ubiYLacZ* reporter gene (as in Figure 6) were synchronized with α-factor for 2 h. Following release from the block, mRNA was prepared from the cells at 15 min intervals, resolved on 1% formaldehyde–agarose, blotted onto nitrocellulose filters and probed with random-primed ^{32}P-labeled probes. **(b)** Hybridization signals corresponding to *CLB2*, *lacZ* and *CLN2* transcripts were quantified by phosphorimaging analysis and plotted over more than two consecutive cell cycles after normalization to the corresponding α-factor signal.

whether Fkh1,2p acted through the defined $CLB2_{UAS}$, *lacZ* mRNAs generated from the $CLB2_{UAS}$–*ubiYLacZ* reporter were also assayed.

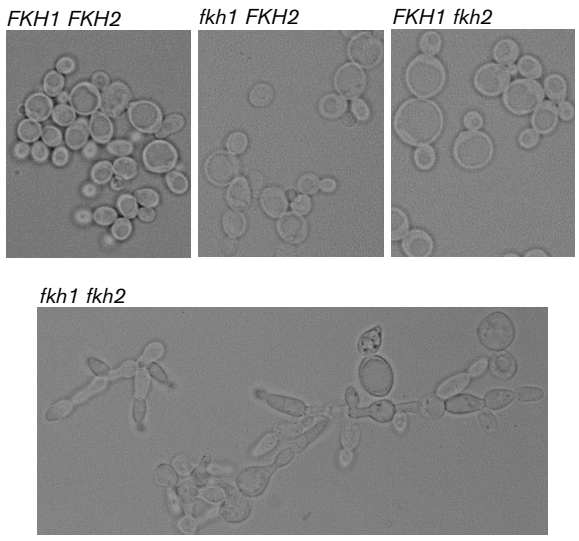
Deletion of either *FKH1* or *FKH2* caused only a modest decline in the absolute levels of *CLB2*, *CDC5*, *SWI5* and *lacZ* transcripts in asynchronous cells, as determined by northern blot analysis (Figure 7a,b). Levels of *CLN2*, *H2A* and *PRT1* mRNAs did not vary significantly in different *FKH* backgrounds. Subtle differences were seen when the levels of these mRNAs were evaluated during synchronous cell cycles. In the case of *CLN2* and *H2A*, there was a slight delay in both activation and repression of transcription in *fkh1* and *fkh2* mutants. This corresponds to a similar delay in the accumulation of *CLB2*, *CDC5*, *SWI5* and *lacZ* transcripts and a delay in progression through the cell cycle, as determined by the kinetics of bud formation following α-factor release (data not shown) and by cell division times. The generation times of *Δfkh1 FKH2* and *FKH1 Δfkh2* strains were approximately 120 minutes, compared to 90 minutes for the isogenic wild-type strain.

Changes in transcription profiles were more dramatic in the *Δfkh1 Δfkh2* double-deletion strain, which displayed a significantly increased generation time (150 minutes), compared with the single *fkh* mutants. Transcripts for *CLB2*, *CDC5*, *SWI5* and *lacZ* were almost extinguished in the double mutant and lost all signs of normal periodicity. In contrast, although *CLN2* and *H2A* transcripts retained the slight delay in activation and repression seen in the single *Δfkh1* and *Δfkh2* mutants, they retained transcriptional periodicity. These data clearly show that *FKH1* and *FKH2* are required for the periodic regulation of CLB2 cluster genes.

Deletion of *FKH1* and *FKH2* results in an altered morphological phenotype

We have shown that deletion of the *FKH1 FKH2* gene pair substantially reduces the levels of CLB2 cluster transcripts, abolishes their periodicity and dramatically lengthens the cell cycle. The accompanying perturbation in cell-cycle progression associated with these defects suggested that this would be manifest in a distinct morphological phenotype, as seen in Clb-deficient strains [17,18]. Microscopic examination revealed that although slightly larger than *FKH1 FKH2* cells, the *Δfkh2* mutant did not display an exaggerated phenotype (Figure 8). The *fkh1* mutant was more comparable in size to the *FKH1 FKH2* strain and, like the *fkh2* mutant, displayed no obvious morphological phenotype. The *Δfkh1 Δfkh2* double mutant, however, displayed obvious morphological changes, the most obvious being a defect in the ability of mother and daughter cells to separate following M phase, resulting in obvious chain-like projections. Double mutants also displayed budding defects, in particular, loss of axial bud growth which resulted in the formation of extended buds.

Figure 8



Morphological phenotype of *Δfkh* mutants. Yeast strains were grown on YEP supplemented with 2% glucose to mid-log phase, mildly sonicated and photographed on a microscope slide (60× objective).

Similar chain-like projections were also observed radiating from colonies grown on agar plates (data not shown).

Discussion

Roles for forkhead proteins in G2–M transcription

We show here that a major component of the SFF activity previously described in cell extracts [3,5] is the forkhead transcription factor Fkh2p. The winged-helix forkhead class of transcription factors are identified by a monomeric DNA-binding domain of 100 amino acids. Since the original identification of the *Drosophila Fork head* mutation, more than 100 family members have been identified in species from yeast to human [19]. These factors have roles in cell-type determination, differentiation, development and cell death, and now, as described here, a new role in cell-cycle regulation.

On examination of the consensus binding site for SFF that is present in *CLB2* and *SWI5* promoters (5′-GTC/AAA-CAA-3′), it appears identical to that reported for forkhead transcription factors in other species [19]. Out of 35 CLB2 cluster genes originally identified by microarray analysis, 26 have easily identifiable binding sites for Mcm1p–SFF/FKH in their proximal promoter regions [2]. This suggests that Mcm1p–forkhead complexes may be common to the overall regulation of CLB2 cluster genes. The relationship between the DNA-binding specificity of SFF and forkhead proteins is underscored by previous observations that SFF makes specific base contacts with a cytosine residue at position 296 in the *SWI5* promoter *in vitro* [3]. This residue is part of the consensus forkhead-recognition site and, if mutated to an adenine (A296), SFF/Fkh2p DNA-binding activity is lost and *SWI5* transcription is abolished [3].

Although recombinant Fkh2p can, by itself, generate ternary complexes indistinguishable from those characterized in cell extracts (Figure 2), we cannot exclude the possibility that other binding activities are also part of this complex. It is interesting that, unlike most other forkhead transcription factors characterized so far, Fkh2p does not bind the DNA recognition site described here as a monomer, but instead as an accessory protein for Mcm1p. The exact role of Mcm1p in ternary complex formation remains unclear. Does it recruit Fkh2p by protein–protein interactions in the same way that it does for other accessory proteins, or does it enforce a DNA conformation more favorable to Fkh2p binding? This latter point is relevant, because DNA bending is thought to influence the ability of the human FREAC forkhead protein to bind its target sequence [20]. The best evidence for the first possibility is the observation that SFF can stabilize the binding of Mcm1p to mutant *SWI5* binding sites ($SWI5_{A309/T308}$) that normally do not support autonomous binding of Mcm1p homodimers [3].

We have also shown that a closely related forkhead family member, Fkh1p, has an overlapping role with Fkh2p in the control of G2–M transcription and specifically binds the *CLB2* promoter *in vivo*. Whereas deletion of *FKH1* or *FKH2* singly had only modest effects on the activity of target genes such as *CDC5*, *SWI5* and *CLB2*, the double mutant strain had significantly reduced levels of these transcripts with an accompanying loss of cell-cycle regulation. Similarly, the cell-cycle-regulated $SWI5_{UAS}$ and $CLB2_{UAS}$ elements, which are necessary and sufficient for cell-cycle-regulated reporter gene activity, are affected in a similar way, showing that Fkh1p and Fkh2p are acting through the Mcm1p-dependent UAS elements. This confirms our general hypothesis that G2–M transcription *in vivo* requires the assembly of Mcm1p–forkhead ternary complexes on cell-cycle-regulated promoters.

Our data show that *FKH1* and *FKH2* are important regulators of the CLB2 cluster and are important both in determination of absolute transcriptional activity and the establishment of periodicity. Unlike *MCM1*, however, *FKH1* and *FKH2* are nonessential genes, and although they have key roles in regulating G2–M transcription, this function is not absolutely required for cell viability. This is especially surprising considering the magnitude of the effect on G2–M transcription in the *Δfkh1 Δfkh2* strain. Similar reductions in transcription of G2–M-regulated genes have been described in cells depleted of Mcm1p, the partner of Fkh1p,2p. In this case, however, cells do

not pass through M phase, presumably because genes required for the G2–M transition are not activated [4]. If the major pathway by which CLB2 cluster genes are regulated is through the Mcm1p–Fkh1/2p complex, why is the role of Mcm1p more critical than the combined roles of Fkh1p and Fkh2p? The answer could be associated with the ability of Mcm1p to recruit additional *trans*-activating factors to promoters in the absence of Fkh1p and Fkh2p. This could explain the low level of constitutive transcription observed in the *Δfkh1 Δfkh2* mutant. Other forkhead genes, such as *HCM1* and *FHL1* [16], have been identified in the *Saccharomyces* genome, and might be candidates for such a scenario. Also, other activities are likely to be able to compensate functionally for reduced expression of some target genes in the absence of forkhead proteins. *CLB3* and *CLB4*, for example, can partially compensate in cells lacking either *CLB1* or *CLB2* [18]. Furthermore, *Δclb3,4* mutants amplify the G2–M cell-cycle arrest phenotype in Mcm1p-depleted cells, suggesting that *CLB3,4* can functionally compensate for defects in the Mcm1p–forkhead regulatory system. If this is correct, deletion of *CLB3* and/or *CLB4* in a *Δfkh1 Δfkh2* mutant should block progression from G2 into M phase. Finally, an alternative explanation is that the Mcm1p–forkhead pathway is not essential and that Mcm1p has, as yet undefined, forkhead-independent functions required for mitotic entry. For example, other Mcm1p-dependent transcriptional pathways, acting independently of forkhead transcription factors, may also be required for mitotic progression.

Forkhead target genes

Spellman and co-workers [2] have identified more than 30 genes that peak in their transcriptional activity during the G2–M transition. Many of these genes are involved, or implicated, in events relating to progression through M phase. Several functional classes of genes thought to be regulated by the Mcm1p–SFF pathway include those involved in bud formation (*BUD4* [21]), mitotic entry (*CLB1,2* [18]), mitotic progression (*CDC20* [20]) and exit (*SWI5* [22]), cytokinesis (*CDC5* [23]), chromosome segregation (*ASE1* [24]) and cell wall biosynthesis. Interestingly, the *Δfkh1 Δfkh2* strain exhibits cell separation defects, delayed entry into mitosis and loss of axial bud growth, and is particularly susceptible to lysis under hypotonic conditions. All these phenotypes are consistent with defects due to decreased activity of the CLB2 cluster. Some of the phenotypes we describe for *fkh1 fkh2* mutants are similar to that described previously for Clb-deficient cells [14,18] and for various alleles of *CDC28/CDK1* [17].

Regulation of Mcm1p–forkhead transcription factor complexes

It will now be crucial to determine how the Mcm1p–forkhead complex activates target genes late in the cell cycle. The DNA-binding activity of Mcm1p, and DNA-binding activities on the *CLB2* and *SWI5* SFF/FKH sites *in vivo*, do not appear to vary throughout the cell cycle [4]. It is, however, not known if the same complex persists throughout the cell cycle or if there is an exchange of activator/repressor factors. Changes in DNA-binding activities cannot therefore be eliminated as a factor in the control of these genes at this stage.

In the absence of SFF binding, Mcm1p does not appreciably activate the *SWI5* or *CLB2* promoter *in vivo* [3–5]. Hence, it is likely that factors other than Mcm1p provide the *trans*-activation function required for switching on genes during G2–M. This function could be provided by forkhead proteins or by Ndd1p, a factor previously implicated in regulation of G2–M transcription [10]. It has been proposed that the activation and maintenance of *CLB2* transcription is dependent upon Clb-associated Cdk activity [14] and that Clb1,2-associated kinases are involved through a positive feedback loop in which they are required for the activation and maintenance of *CLB* transcription. This model fits well with the kinetics of *CLB2* transcription and Clb2-associated kinase activities and offers an explanation as to how *CLB2* transcription is shut down at the end of mitosis, when Clb kinase becomes inactive. The presence of six canonical Cdk phosphorylation sites in the carboxy-terminal region of Fkh2p is interesting and is a possible link between Cdc28p–Clbp activity and the transcriptional control of *CLB2* and other co-regulated genes.

Are G2–M transcriptional control mechanisms conserved in eukaryotes?

Although cell-cycle-regulated transcriptional pathways are only loosely conserved during evolution, it is interesting that the *sep1(+)* gene from fission yeast encodes a forkhead transcription factor that is required for cell separation and causes a hyphal phenotype when deleted [25]. It is not known, however, if sep1 functions in collaboration with map1, a MADS box transcription factor required for cell-type determination in fission yeast [26,27]. Given that the molecular basis of cell-cycle control is fundamentally conserved between unicellular yeast and metazoans, this raises the question of whether the Mcm1p–forkhead mode of G2–M transcriptional control also functions in higher eukaryotes.

Mammalian counterparts of Mcm1p function by recruiting specific accessory proteins in a similar way to Mcm1p in yeast, through a domain adjacent to the MADS box [6]. The best example is the mammalian serum-response factor (SRF), which recruits ternary complex factors to the c-*fos* serum-response element via protein–protein interactions [6,28]. It is not known if mammalian factors such as SRF can function with forkhead proteins, but when the DNA-binding specificity of SRF is changed to that of Mcm1p, it can substitute functionally for Mcm1p in yeast [11]. In some contexts, then, SRF or other MADS box transcription factors might interact with forkhead family members.

In summary, we have shown that forkhead transcription factors collaborate with Mcm1p to coordinate the activation of a program of transcription specifically required for mitosis and cytokinesis. Future work will focus on the molecular mechanisms that control the activity of this regulatory pathway.

Materials and methods

Strains, media, reagents and yeast genetic protocols

All yeast strains were derivatives of W303 (*MAT**a*** or *MATα ho ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 ssd1*) except where otherwise indicated. Yeast strains were grown in YEP medium (1% yeast extract, 2% Bacto-peptone, 100 mg/l adenine) supplemented with 2% glucose (YEPD), raffinose (YEPR) or raffinose plus galactose (YEPG). All $CLB2_{UAS}$–*ubiYLacZ* reporter strains have been described [5]. Single-copy integrations were confirmed by Southern blot or PCR analysis. Gene disruptions, yeast transformations and other yeast techniques were by standard methods. Liquid culture and qualitative plate-based assays used to determine β-galactosidase activity were as described [28]. *GUS* reporter gene activity was evaluated by a modification to a described method [29].

Purification of SFF activity and mass spectrometry

A 20 l culture (90 g wet weight) of the strain S226 (SL3-10B*: MAT**a** mcm1::LEU2 ADH-mcm1*$_{1-98}$*::URA3*, otherwise isogenic with *W303* [11]) was grown in YEPD to OD_{600} = 0.8. Cell extracts were prepared essentially as described by Ammerer [30]. Affinity beads were generated by concatenating $SWI5_{UAS}$ oligonucleotide duplexes (sequence available on request [3]), to create a hexamer that was inserted into the *Sma*I site of pBS.KS$^+$. This fragment was then PCR amplified using oligonucleotides 5′-CGAGGTCGACGGTATCG-3′ and 5′-biotin-GCCGCTCTAGAACTAG-3′, which prime at positions immediately flanking the hexamer insert. The biotinylated $SWI5_{UAS6}$ fragment was gel purified and coupled to 10 ml streptavidin–agarose beads (Sigma) in the presence of 1× binding and washing buffer (1× BWB: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1.0 M NaCl), under conditions where the DNA-binding capacity of the beads was saturated. Mcm1p–SFF complexes were purified at room temperature unless otherwise stated. For a typical purification, 10 ml extract (20–25 mg protein per ml) was diluted in 40 ml binding buffer (1× BB: 20 mM Tris-HCl pH 7.5, 20 mM EDTA pH 8.0, 10% glycerol, 1 mM DTT, 0.01% Nonidet-P40, 50 mM NaCl, 3 mM $MgCl_2$, 50 μg/ml BSA, 5 mM spermidine, 50 μg/ml sonicated herring sperm DNA, 1 mM benzamidine, 1 μg/ml leupeptin, 1 mM PMSF, 1 μg/ml aprotinin) and centrifuged at 18,000 rpm in a Sorval SS34 rotor at 4°C. The supernatant was then tumbled with 10 ml charged DNA affinity beads (50% v/v slurry) for 15 min and poured into a BioRad EconoColumn. Unbound proteins were eluted with 50 ml 1× BB in 250 mM NaCl under gravity. Polypeptides retained in the column were eluted with 1× BB in 700 mM NaCl and assayed for activity by band-shift analysis. Fractions containing Mcm1p and SFF activity were dialyzed against 1× BB, affinity purified a second time under the same conditions and concentrated in Centricon 10 spin columns (Amicon) before final purification on 8% SDS–PAGE.

Proteins separated by one-dimensional PAGE were visualised by silver staining and then excised from the gel [31]. The protein of interest, migrating with an apparent molecular weight of 98 kDa, was reduced *in situ* with DTT, alkylated with iodoacetamide and digested with trypsin (Boehringer Mannheim, unmodified, sequencing grade) as described [31]. Proteins were identified by MALDI mass spectrometry on a REFLEX mass spectrometer (Bruker Daltonics) [32].

Plasmid constructions

The *FKH1* and *FKH2* ORFs were generated by PCR using yeast genomic DNA as template and inserted into plasmids as described below. All DNA fragments generated by PCR were confirmed by DNA sequencing. All constructs used for *in vitro* transcription–translation analysis were subcloned into pT7βpLink [28]. Full construction details are available from the authors.

pT7.FKH2$_{1-862}$: 5′-(*Nco*I)CC.ATG.GCC.(FKH2 codons 3–862).CCA.AGC.GGC.CGC.TGA. CTCGAG(*Xho* I)-3′. *pVP16–FKH2* was constructed by inserting a *FKH2* PCR fragment into the galactose-inducible expression plasmid p4064 [33]: 5′-GAL_{1-10}/*CYC1* promoter($SV40_{NLS}$)(*myc* tag)($Vp16_{410-490}$) G.AAT.TCC.ATG.GCC.(*FKH2* codons 3–457).TGA.CTCGAGATCGATTAGACTAGATATC–*CYC1* terminator-3′ (*Eco*RI–*Spe*I *FKH2* fragment into p4064 cut with *Eco*RI–*Xba*I).

The *FKH1* and *FKH2* ORFs were epitope-tagged by inserting a *Not*I restriction fragment containing six *myc* tags into a *Not*I site immediately before the STOP codon (see pT7.FKH2$_{1-862}$). Full-length *FKH2–MYC* was placed under galactose-inducible control (*GAL1* promoter) by subcloning into the *Eco*RI–*Xho*I sites of pYES2. To generate a vector for integration at the *FKH1,2* loci, restriction fragments were subcloned into YIplac204.

Designating the first nucleotide position of the ATG initiator codon as +1, integration constructs were as follows. YIpFKH1–MYC: *Bam*HI-(732)G.GAT.CC(*FKH1)*CTG.AGT(1447)*Not*I(myc_6)*Not*ITGA.CTCGAG *Xho*I, linearized with *Hin*cII for integration at *FKH1*. YIpFKH2–MYC: *Hin*dIII(968)AA.GCT.T(*FKH2)*AAC.AAC(2586)*Not*I(myc_6)*Not*ITGA.CT CGAG*Xho*I, linearized with *Kpn*I for integration at *FKH2*.

Disruption constructs were made as follows. FKH1: a *FKH1 Bsp*H1–*Xho*I PCR fragment spanning the complete ORF was subcloned into pT7βpLink. This construct was then opened-up with *Sna*BI–*Bsp*MI, blunted and used as a recipient for a blunt-ended *LEU2* gene. This construct was then used as a template for PCR, generating a *FKH1–LEU2–FKH2* product spanning from the initiator ATG to the termination codon of *FKH1*, with intervening *LEU2* sequences. After purification this was used directly in yeast transformations. FKH2: A PCR fragment from the *Xba*I site in the *FKH2* ORF (358) to the STOP codon (5′-TGA,CTCGAG-3′) was inserted into pBS.KS$^+$ cut with *Xba*I–*Xho*I. This construct was opened up with *Hin*dIII–*Bam*HI, blunted, and used as a recipient for a blunt-ended *URA3* gene fragment. This construct was digested with *Xba*I–*Kpn*I, gel purified and transformed into yeast.

Band-shift assays, chromatin immunoprecipitation, northern blot hybridization and cell synchronization

Preparation of cell extracts, band-shift analysis, band-shift probes, northern blot hybridization, DNA probes, and α-factor synchronization have all been described previously [3,5]. Recombinant Mcm1p$_{1-112}$ (rMcm1p$_{1-112}$) was produced by inserting an *Nco*I–*Bam*HI-cut PCR product, encoding His_6-tagged Mcm1$_{1-112}$, into pET15b (Novagen) to form p6His.MCM1$_{1-112}$: (pET15b)5′-CC.ATG.GCA.(*MCM1* codons 1–112)His_6.STOP.GGATCC-3′. This was transformed into *BL21 pLysS* and the recombinant protein purified under non-denaturing conditions on Ni–agarose beads (Qiagen) according to the manufacturer's instructions. Chromatin immunoprecipitation assays were performed essentially as described [34].

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